Differential expression analysis of *Liprin-α2* in hibernating bat (*Rhinolophus ferrumequinum*)

Lihong Yuan [a,b,c], Xueguo Zuo [a], Jinping Chen [d], Benfu Lin [e], Junpeng Zhang [b], Min Sun [e], Shuyi Zhang [a,*]

[a] *School of Life Science, East China Normal University, Shanghai 200062, China*
[b] *Institute of Zoology, Chinese Academy of Sciences, Beijing 100080, China*
[c] *Graduate University of Chinese Academy of Sciences, Beijing 100080, China*
[d] *Guangdong Entomological Institute, Guangzhou 510260, China*
[e] *Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou 510663, China*

Abstract

A PCR-based subtractive hybridization technique was used to identify genes up-regulated in the hibernating bat brain to explore the molecular mechanism of hibernation. Three genes, *Liprin-α2*, *PTP4A2* and *CAMKKβ* were differentially expressed in hibernating bat brain tissue compared to active bat brain tissue. One of them, *Liprin-α2*, which has recently been shown to have the key function in the organization of presynaptic and postsynaptic multiprotein complexes was studied in detail. We demonstrated that the expression level of *Liprin-α2* was up-regulated almost 4-fold in hibernating bat brains by RT-PCR compared to levels in active bats. The differential expression pattern of *Liprin-α2* was also detected in muscle, fat, brain and heart tissue of hibernating bats by real-time quantitative PCR. The result indicated that *Liprin-α2* was over-expressed in brain and heart tissue and down-regulated in muscle and fat. In brain tissue of hibernating bats, *Liprin-α2* expression was statistically significantly higher than in brain tissue of active controls ($P = 0.029$). The precise control of transcriptional level and the distinctively differential expression pattern of *Liprin-α2* in different organs during circannual hibernation may have important physiological significance, not only in maintaining normal function of many key organs but also in effectively conserving limited energy resources without physiological damage.

Keywords: Bat; Differential expression; Hibernation; *Liprin-α2*

1. Introduction

Many small mammals can survive in harsh winter weather by hibernation. The prolonged bouts of torpor involved are interrupted by brief periodic arousals with spontaneously rewarming [1–5]. During torpor, metabolic rate may be reduced to 1–5% of the normal euthermic rate, and core body temperature can fall to 0–5 °C. Data suggest that by hibernating, hibernators can save nearly 90% of the energy that they would otherwise require to remain euthermic over the winter months [6]. Although it has already been shown that with the exception for hypothermia [7], animals are also protected from ischemia-reperfusion injuries [8,9], muscle disuse [10], bacterial infection [11] and carcinogenesis [12] during hibernation, little is known about the underlying molecular mechanisms. Studies indicate that many molecular mechanisms coordinate entrance into and arousal from torpor, including reversible phosphorylation of key enzymes and functional proteins and the selected differential expression of genes [9,13–15].

Greater horseshoe bat (*Rhinolophus ferrumequinum*) is a kind of small mammalian, which hibernates annually and has prolific resource in China, and the brain, as the key component of the central nervous system, plays an important regulating role during hibernation [16]. Several brain regions, such as the hippocampus, septum, hypothalamus and suprachiasmatic nucleus, may be involved in the central control of hibernation [17–19]. In this study, a suppression subtractive hybridization (SSH) library of the hibernating bat brain was constructed for exploring the molecular mechanism of hibernation, and up-regulation genes in bat brains of hibernating state were obtained by PCR-based subtractive hybridization. In them, *Liprin-α2* was chosen to further investigate the differential expression patterns in different tissues of bats in a hibernating state.

2. Materials and methods

2.1. Animals and RNA extraction

Eight greater horseshoe bats were captured from caves (39°48′N, 115°42′E) in Fangshan area of Beijing, China in the winter of 2004 and the summer of 2005. In late November 2004, four greater horseshoe bats, which had been hibernating for almost one month and the range of rectal temperatures of them were 8.7–11.8 °C, were captured and sacrificed before waking from hibernation state, and four kinds of tissues (whole brain, white adipose tissue (WAT), heart and skeletal muscle) were rapidly excised and conserved in liquid nitrogen. In mid-June 2005, four flying greater horseshoe bats were netted and sacrificed immediately, and four tissues were rapidly excised and flash-frozen in liquid nitrogen. All tissue samples were transported and stored in −80 °C until used for RNA extraction. Total RNA was isolated from tissues of eight greater horseshoe bats using the RNAiso kit (TakaRa, Japan), and quantified by the ratio of OD_{260}/OD_{280}. The quality of RNA isolated was detected by agarose gel electrophoresis.

2.2. PCR-based subtractive hybridization (SSH)

Total RNA of three hibernating bat brains and three active bat brains (2 μg each) were mixed, respectively, and poly(A) RNA were purified by using PolyATtract® mRNA Isolation Kit (Promega). The poly(A) RNA of hibernating and active bats were used as templates, and double-strand cDNAs were synthesized by Super SMART™ PCR cDNA Synthesis Kit (Clontech). After digested by RasI, the double-strand cDNA of hibernating state and aroused state served as tester and driver, respectively. Tester cDNA was aliquotted into two separate parts and ligated with adaptor 1 and 2, respectively, and hybridized by the driver cDNA twice. In the first hybridization, an excess of driver cDNA was added to each sample of tester, and the samples were then heat-denatured and allowed to anneal. During the second hybridization, the two primary hybridization samples were mixed together, and fresh denatured driver cDNA was added to further enrich the differentially expressed sequences in hibernating bat brains. Successively, nest PCR was performed to amplify the desired differentially expressed sequences. Amplified cDNAs were resolved on 2% agarose gels and visualized by SYBR GreenI staining to confirm successful subtraction.

2.3. Cloning and differential hybridization screening of subtracted cDNAs

Subtracted cDNAs were cloned into PGEM-T easy vector (Promega) and clones containing inserts were selected by blue/white screening assay. In order to reduce the number of false positive subtracted cDNAs, clones containing inserts were further screened by hybridisation using the PCR-Select™ cDNA Subtraction Kit (Clontech). Dot blot was carried out to screen highly expressed genes in brain tissues of hibernating bats with biotin-labeled cDNA probe (SpotLight™ Random Primer Labeling Kit, Clontech), and the positive clones were sequenced and analyzed using the BLAST network service at NCBI.

2.4. Semi-quantitative RT-PCR analysis

Brain tissues from four hibernating bats and four active bats were used to synthesize cDNA. Two microgram of total RNA from each sample was treated with 2 U of RNase-free DNase I (Promega) for 30 min at 37 °C to avoid genomic DNA contamination, then converted to cDNA by SuperScrip III Reverse Transcriptase (Invitrogen) following the manufacturer's instruction, which contained 500 ng of random primer, 1 mM dNTP, 2 mM dithiothreitol, 80 U RNase inhibitor (Promega), 1× first-strand buffer and 400 U SuperScrip III reverse transcriptase in a 50 μL reaction mixture. The PCR cycle number required for amplification to be within the exponential phase was experimentally determined for each primer pair. First-strand cDNAs were normalized with respect to expression of the housekeeping gene β-actin (forward primer: 5′ GAC CTC TAT GCC AAC ACA G 3′, reverse primer: 5′ CAT CTG CTG GAA GGT GGA CA 3′) and subsequently used to assess *Liprin-α2* expression levels (sense primer: 5′ GTT TAT CTG CCT CGC TTG 3′; anti-sense primer: 5′ TGA TTC CTT TCT TCT TGG G 3′). PCR was performed with the condition of 94 °C predenature for 5 min, 30 cycles of 94 °C for 30 s, 53 °C for 30 s, 72 °C for 2 min and finally 72 °C for 10 min. The products were resolved on 3% agarose gels and visualized by SYBR GreenI staining.

2.5. Differential expression analysis of Liprin-α2 in hibernating and active states bats

Four tissues (brain, heart, muscle and fat) from four hibernating greater horseshoe bats and four active greater horseshoe bats were used as templates (dilution 1: 10). Primers of *Liprin-α2* and β-actin used for the real-time

quantitative-PCR (RQ-PCR) were the same with those in RT-PCR analysis. RQ-PCR was performed using PTC-200 (MJ Research) and the fluorescence threshold value was calculated using Opticon2.0 system software. PCR was performed by the two-step method with the following conditions: pre-denaturation at 94 °C for 1 min followed by 45 cycles of 94 °C for 10 s, 53 °C for 20 s. The plate was read and the melting curve was generated using a 20 μL PCR mixture containing 10 μL SYBR Green I Premix Ex *Taq* (TakaRa), 2.5 μL cDNA template and 0.4 μM of each primer. The $2^{-\Delta\Delta C_T}$ method was used for quantity calculations [20].

All data were expressed as means and analyzed by Mann–Whitney test to determine the statistical significance of the data by SPSS software 10.0. $P < 0.05$ was taken to represent a statistically significant difference between group.

3. Results

3.1. Construction and screening of the bat brain SSH library

Total RNA was also electrophoresed on a 1% agarose gel to assess quality visually (Fig. 1). After construction and subtraction of the bat brain SSH library, significant differences were observed in the subtracted cDNA pools as compared to the unsubtracted cDNA pools, indicating successful subtraction (data not shown). Subtracted cDNAs were cloned into pGEM-T vector and differential screening was used to further reduce the number of false positive cDNAs common to both tester and driver populations. If the signal produced was more than three times the background, a hybridized spot was considered to have a positive cross-reaction, and genes were considered up-regulated if there was a 2-fold or more difference when comparing the hibernating signal with that of the active one. Clones remaining positive after differential screening were sequenced and the sequences obtained were compared with sequences in the GenBank, EMBL and dbEST databases. Three candidate genes were obtained including *Liprin-α2*, *PTP4A2* and *CAMKKβ* [21]. Sequence of greater horseshoe bat *Liprin-α2* gene was submitted to NCBI database (Accession Nos. EF026107).

3.2. Differential expression analysis of Liprin-α2 in hibernating bat

Due to its important role of *Liprin-α2* in regulating the formation and/or maintenance of presynaptic active zones

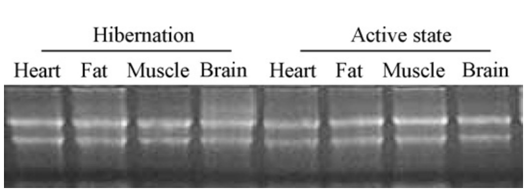

Fig. 1. Total RNA detected by agarose gel.

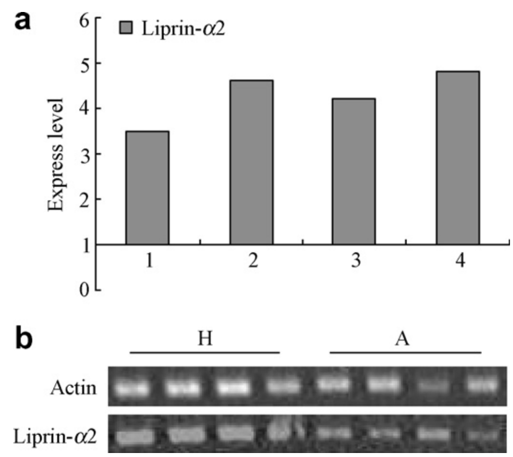

Fig. 2. Relative transcript levels of Liprin-α2 in hibernating and active bat brains detected by reverse-transcript PCR. (a) The axis of ordinates indicates the relative mRNA levels of Liprin-α2 (hibernating versus active) in bat brains. The axis of abscissas indicate that the reaction was repeated four times in different individual. (b) RT-PCR products detected by agarose gels. H, hibernating state; A, active state.

and postsynaptic targeting of AMPA (alpha-amino-3-hydroxy-5-methyl-4-isooxazole proprionic acid) receptors, *Liprin-α2* was chosen to further analyze the differential expression pattern in hibernating and active states of bats.

RT-PCR was performed to analyze the up-regulation level of *Liprin-α2* in hibernating bat brains and repeated four times in different individuals in order to reduce intra-individual differences. Results showed that the average expression level of *Liprin-α2* in bat brains was increased nearly 4-fold in hibernating versus active states (Fig. 2). RQ-PCR was carried out to identify the expression patterns of *Liprin-α2* in four key tissues of bats during hibernation. Data indicated distinctively different expression patterns among the four tissue types. In heart and brain, the transcript level of *Liprin-α2* increased 1-fold and 3.7-fold, respectively, but decreased 27% and 36% in fats and muscles. Statistical analysis suggested that the up-regulation of *Liprin-α2* in hibernating bat brains was statistically significant in comparison with the active state ($P = 0.029$, $P < 0.05$) (Table 1 and Fig. 3).

4. Discussion

Liprin-α2 is a member of Liprin-α family, a multidomain protein family consisting of four isoforms [22,23]. By interacting with the LAR (gene symbol PTPRF) family of receptor protein tyrosine phosphatases and the GRIP/ABP family of AMPA receptor-interacting proteins, Liprin-α is involved in the regulation of the development of presynaptic zones and postsynaptic targeting of AMPA receptors [22,24,25]. Studies showed that a mutation in *Caenorhabditis elegans* homolog of Liprin-α, synapse-defective 2, resulted in lengthening of presynaptic active zones and impaired synaptic transmission [26]. Furthermore, mutations in both Dliprin-α and Dlar (*Drosophila*

Table 1
Relative transcript levels of bat *Liprin-α2* in different tissues and different states detected by real-time quantity PCR

	Heat				Fat (WAT)				Whole brain				Skeletal muscle			
	Actin	Liprin	ΔC_T	$2^{-\Delta\Delta C_T}$	Actin	Liprin	ΔC_T	$2^{-\Delta\Delta C_T}$	Actin	Liprin	ΔC_T	$2^{-\Delta\Delta C_T}$	Actin	Liprin	ΔC_T	$2^{-\Delta\Delta C_T}$
H[a]	23.174	31.534	8.36		20.499	31.297	10.798		21.705	25.527	3.822		19.678	32.741	13.063	
	24.022	31.778	7.756		20.194	31.002	10.808		21.464	26.367	4.903		23.39	33.735	10.345	
	24.357	32.156	7.799		20.874	30.993	10.119		21.297	25.323	4.026		21.141	33.209	12.068	
	23.891	31.707	7.816		20.192	32.002	11.81		21.485	25.746	4.261		21.411	33.236	11.825	
Avg[c]	23.861	31.794	7.933	2.174	20.440	31.324	10.885	0.725	21.488	25.741	4.253	4.706	21.405	33.230	11.825	0.646
A[b]	24.222	32.342	8.12		20.179	31.879	11.7		23.76	31.601	7.841		22.775	34.313	11.538	
	22.425	32.07	9.645		24.39	34.088	9.698		25.684	32.216	6.532		21.317	33.727	12.41	
	22.656	32.654	9.998		24.772	34.3	9.528		25.053	30.141	5.088		23.137	32.765	9.628	
	23.912	32.812	8.449		23.192	33.945	10.753		24.841	31.33	6.489		22.415	33.616	11.201	
Avg[c]	23.304	32.469	9.053	1	23.133	33.553	10.420	1	24.835	31.322	6.488	1	22.411	33.605	11.194	1
P			0.057				0.343				0.029*				0.486	

Data were dealt with $2^{-\Delta\Delta C_T}$ method and all of them have been performed a Mann–Whitney test for homogeneity of variances along with nonparametric tests. Asterisk indicates significant difference ($P < 0.05$). [a]Hibernation; [b]active state; [c]average.

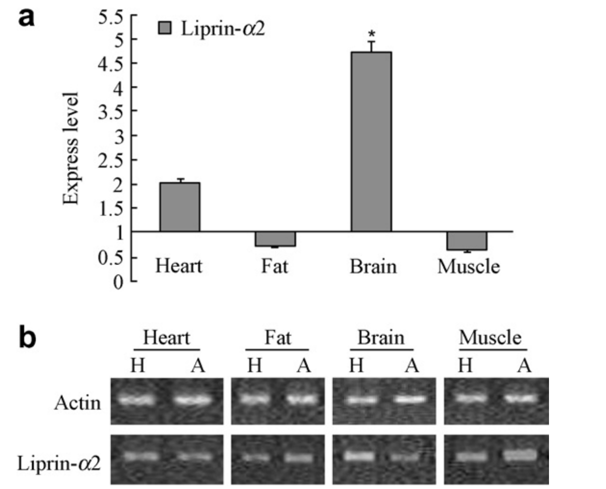

Fig. 3. Relative transcript levels of bat Liprin-α2 in different tissues and different states. (a) Asterisk indicates significant difference ($P < 0.05$) and (b) a part of RQ-PCR products detected by agarose gels. H, hibernating state; A, active state.

homologs of Liprin-α and LAR) led to defects in axon terminal branching and active zone dimensions [27]. Ko et al. reported that the GTPase-activating protein (GIT1) can directly interact with Liprin-α [28]. GIT1, as a multidomain protein with GTPase-activating protein activity for the ADP-ribosylation factor family of small GTPase, can regulate protein trafficking and the actin cytoskeleton [29]. Because of distribution of GIT1 in the region of postsynaptic density (PSD) and presynaptic active zones, GIT1 forms a complex with Liprin-α in the brain and may play an important role in the organization of presynaptic and postsynaptic multiprotein complexes [28]. At the same time, Ko et al. [28] reported that Liprin-α can also directly interact with the ELKS-Rab6-interacting protein-CAST (ERC) family of proteins, which are known to bind Rab3-interacting molecules (RIMs), active zone components that regulate neurotransmitter release [25]. The interaction between Liprin-α and ERC may be involved in the presynaptic localization of Liprin-α and the molecular organization of presynaptic active zones [28].

Recently, the method of screening cDNA library was used extensively to study hibernation-responsive gene expression in several animals and organs, and many differentially expressed genes in hibernating state were identified [4,30–33]. Subtractive hybridization methods are widely used to isolate up- or down-regulated genes [34]. The brain plays an important role in adaptation to hibernation, and many brain regions are shown to be involved in the central control of hibernation [17–19] and the avoidance of neurological damage [8,35–37]. *Liprin-α2* was up-regulated in bat brains in a hibernating state and obtained by PCR-based subtractive hybridization. As heart, fat and muscle are other key organs except for brain and have important functions in circulation of nutrient substance and oxygen during hibernation [9,37], RT-PCR and RQ-PCR analyses were performed to analyze the expressed pattern of

Liprin-α2 in different tissues during hibernation. Data showed that the expression level of *Liprin-α2* in hibernating brains increased 3.7-fold and was statistically significant at the 0.05 level ($P = 0.029$) (Table 1 and Fig. 3). This suggested that *Liprin-α2* may play an important role in the adaptive regulation of brain and neuroprotective properties during hibernation. Further differential expression analysis showed that *Liprin-α2* exhibited a distinct expression pattern in different tissues in a hibernating state (Table 1 and Fig. 3). The expression level of *Liprin-α2* increased about 1-fold in the heart during hibernation, as the important function of *Liprin-α2* played in signal pathway, indicating that *Liprin-α2* may be involved in regulating blood circulation. However, the transcript level of *Liprin-α2* down-regulated 27% and 36% in fat and muscle, respectively, during hibernation. Though the differentially expressed level of *Liprin-α2*, in heart, fat and muscle, was not significant and this may be due to the lack of statistical power stemming from the small sample size. The observed differential expression of selected genes may be understood as an efficient means of reducing energy expenditure and maintaining the basic needs of life during a long period of hibernation.

The precise control of transcriptional level of *Liprin-α2* and the differential expression pattern in different organs during circannual hibernation have important physiological significance, not only in maintaining normal function of many key organs but also in effectively conserving limited energy resources without physiological damage. The mechanism of differential expression of *Liprin-α2*, however, remains unclear and further investigation is needed to clarify this process.

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